Data Analysis Sample Report

# Metabolomics of Drug-Treated vs. Wild-Type Mice


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# Project Summary

### Sample description:

Ten mouse blood samples were received. Five mice were drug treated while the other five were treated with a vehicle control.

### Goal:

To identify and characterize the metabolic changes associated with drug treatment.

### Assay summary:

Global metabolomics assays were performed on all mouse samples, including polar and lipid fractions analyzed with both positive and negative ionization.

### Analysis summary:

Global metabolomics data was processed with unbiased peak characterization and metabolite identification. Statistical analysis was performed to identify metabolites with significant alterations with drug treatment. Results were aggregated through pathway analysis.

### Conclusions:

The results of the analysis show substantial metabolic alterations with drug treatment. In particular, sugar metabolism pathways showed significant disruption. We recommend future studies targeted towards metabolic enzymes associated with sugar metabolism pathways to elucidate mechanisms of metabolic alterations.

# Experimental Methods

## Sample preparation

### Sample handling and storage

Plasma was isolated from blood samples (collected in EDTA tubes) through centrifugation at 200 x g for 10 minutes at room temperature, removal of plasma layer, and centrifugation of plasma at 1,000 x g for 10 minutes. The supernatant was kept and diluted 1:4 with methanol (v/v), vortexed for 30 s, and incubated at -20°C for 2 hours. Samples were centrifuged for 10 minutes at 13,500 x g at 4°C and supernatant was transferred to a new centrifuge tube, concentrated, and stored at 80°C.

### Polar metabolite extraction

Plasma was thawed on ice. A 50 mL aliquot was transferred onto a solid- phase-extraction (SPE)-system CAPTIVA-EMR Lipid 96-wellplate (Agilent Technologies) before addition of 250 mL of acetonitrile containing 1% formic acid (v/v) and 10 mM internal standard (consisting of uniformly 13C and 15N labeled amino acids from Cambridge Isotope Laboratories, Inc). The samples were mixed for 1 min at 360 rpm on an orbital shaker at room temperature prior to a 10 min incubation period at 4°C. Afterward, 200 mL 80% acetonitrile in water (v/v) was added to the samples. The samples were mixed on an orbital shaker (360 rpm) for an additional 10 min at room temperature. The samples were then eluted into a 96-deepwell collection plate by centrifugation (10 min, 57 x g, 4°C followed by 2 min, 1000 x g, 4°C). Polar eluates were stored at 80°C until the day of LC/MS analysis.

### Lipid metabolite extraction

The SPE-plates from the polar metabolite extraction were washed twice with 500 mL 80% acetonitrile in water (v/v). Lipids still bound to the SPE-material were then released into a second elution plate, in two elution steps applying 2x 500 mL 1:1 methyl tert-butyl ether:methanol (v/v) onto the SPE cartridge and centrifuging for 2 min at 1000 x g and 4°C. The combined eluates were dried under a stream of nitrogen (Biotage SPE Dry Evaporation System) at room temperature and reconstituted with 100 mL 1:1 2-propanol:methanol (v/v) prior to LC/MS analysis.

## Metabolomics assays

### LC/MS analysis of polar metabolites

A 2 mL aliquot of polar metabolite extract was subjected to LC/MS analysis by using an Agilent 1290 Infinity II liquid-chromatography (LC) system coupled to an Agilent 6540 Quadrupole-Time-of-Flight (QTOF) mass spectrometer with a dual Agilent Jet Stream electrospray ionization source. Polar metabolites were separated on a SeQuant ZIC-pHILIC column (100 3 2.1 mm, 5 mm, polymer, Merck-Millipore) including a ZIC-pHILIC guard column (2.1 mm x 20 mm, 5 mm). The column compartment temperature was maintained at 40°C and the flow rate was set to 250 mL/min. The mobile phases consisted of A: 95% water, 5% acetonitrile, 20 mM ammonium bicarbonate, 0.1% ammonium hydroxide solution (25% ammonia in water), 2.5 mM medronic acid, and B: 95% acetonitrile, 5% water, 2.5 mM medronic acid. The following linear gradient was applied: 0 to 1 min, 90% B; 12 min, 35% B; 12.5 to 14.5 min, 25% B; 15 min, 90% B followed by a re-equilibration

phase of 4 min at 400 mL/min and 2 min at 250 ml/min. Metabolites were detected in positive and negative ion mode with the following source parameters: gas temperature 200°C, drying gas flow 10 L/min, nebulizer pressure 44 psi, sheath gas temperature 300°C, sheath gas flow 12 L/min, VCap 3000 V, nozzle voltage 2000 V, Fragmentor 100 V, Skimmer 65 V, Oct 1 RF Vpp 750 V, and m/z range 50-1700. Data were acquired under continuous reference ion mass correction at m/z 121.0509 and 922.0890 for positive ion mode and m/z 119.0363 and 966.0007 for negative ion mode. Samples were randomized prior to analysis. In addition, a quality control sample was injected after every 12th sample to monitor signal stability of the instrument.

### LC/MS analysis of lipid metabolites

A 2 mL aliquot of lipid metabolite extract was subjected to LC/MS analysis by using an Agilent 1290 Infinity II LC-system coupled to an Agilent 6545 QTOF mass spectrometer with a dual Agilent Jet Stream electrospray ionization source. Lipids were separated on an Acquity UPLC HSS T3 column (2.1 3 150 mm, 1.8 mm) including an Acquity UPLC HSS T3 VanGuard Pre-Column (2.1 3 5mm, 1.8 mm) at a temperature of 60°C and a flow rate of 250 mL/min. The mobile phases consisted of A: 60% acetonitrile, 40% water, 0.1% formic acid, 10 mM ammonium formate, 2.5 mM medronic acid, and B: 90% 2-propanol, 10% acetonitrile, 0.1% formic acid, 10 mM ammonium formate (dissolved in 1 mL water). The following linear gradient was used: 0-2 min, 30% B; 17 min, 75% B; 20 min, 85% B; 23-26 min, 100% B; 26 min, 30% B followed by a re-equilibration phase of 5 min. Lipids were detected in positive and negative ion mode with the following source parameters: gas temperature 250°C, drying gas flow 11 L/min, nebulizer pressure 35 psi, sheath gas temperature 300°C, sheath gas flow 12 L/min, VCap 3000 V, nozzle voltage 500 V, Fragmentor 160 V, Skimmer 65 V, Oct 1 RF Vpp 750 V, and m/z range 50-1700. Data were acquired under continuous reference ion mass correction at m/z 121.0509 and 922.0890 in positive ion mode and m/z 119.0363 and 966.0007 in negative ion mode. Samples were randomized before analysis. In addition, a quality-control (QC) sample was injected after every 12th sample to monitor signal stability of the instrument.

## Data preprocessing

### Metabolite detection and identification

Metabolite signals (features) were detected in the LC/MS data through in-house peak detection and curation software. Features were aligned across samples and features with intensities greater than 1/3 of the corresponding intensity in the QC sample were classified as contaminates and removed from future analysis. Feature degeneracy (isotopes, adducts, fragments, etc.) were identified through clustering and ion assignment with in-house software. If present, the (de)protonated ion for each metabolite was kept. Otherwise, the highest observed ion was kept.

Metabolites were structurally identified through searching against online metabolite structure databases (Human Metabolome Database, PubChem, KEGG) and comparing isotope patterns, MS/MS fragmentation data (when available), and predicted retention times. Metabolite identifications were scored and filtered to only keep metabolite identifications with a false discovery rate of <1%.

### Data normalization and curation

Metabolomics data from all assays were concatenated. Missing values were imputed by using half of the minimum detected intensity for each metabolite. To remove technical variation, a random forest model was fit by using the analysis order and batch of each QC sample to predict signal drift at each analysis position. Predicted drift was removed from each sample to minimize technical variation. Metabolite intensities were log2 transformed prior to statistical analysis.

Metabolite identifications and intensities were manually reviewed for concordance and accuracy.

## Statistical analysis

Technical variation was assessed by calculating the coefficients of variation (CVs) for each metabolite from the standard deviation divided by the mean of the non-log2 transformed abundance values for each metabolite in the QC samples.

Hypothesis testing was performed with a two-tailed, two-sample t-test with unequal variance. Log2 normalized metabolite intensities were used for null hypothesis testing. The resulting p-values were corrected with the Benjamini-Hochberg Procedure. Fold-changes were computed from non-log2 intensities.

Over-representation analysis was performed with a Fisher's Exact test comparing the expected number of metabolites found to be significant in each pathway with the number of significant metabolites found in each pathway.

# Results

## Metabolite summary

Across the four assays used to profile the samples, 1,039 metabolites were detected and identified (Figure 1). The relative abundance of each metabolite was quantified across each sample. The metabolites profiled in the polar fraction of the samples were largely composed of organic acids, nucleic acids, and fatty acyls. In the lipid fraction, glycerophospholipids and sphingolipids composed the majority of the profiled metabolites. In the positive-mode lipid data, a large number of glycerolipids were also profiled (Figure 2).

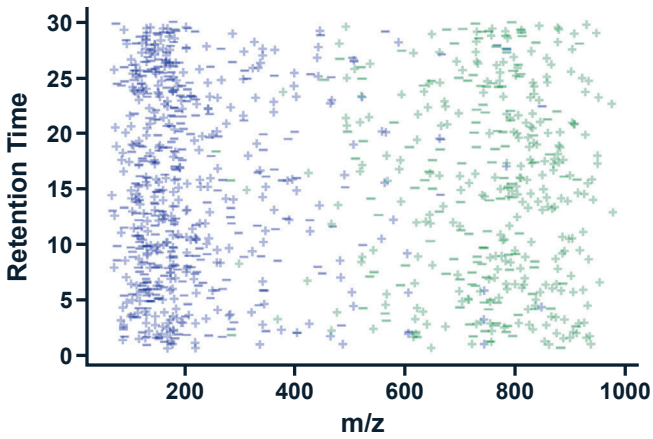


**Figure 1: Detected metabolite signals.** Each metabolite signal is represented by a point on the plot representing the m/z and retention time coordinates of the feature. Color of dots corresponds to the assay type (green = lipid, blue = polar) with the ionization mode indicated by the marker ("-" = negative mode, "+" = positive mode). The size of the points corresponds to the mean intensity of the signal.

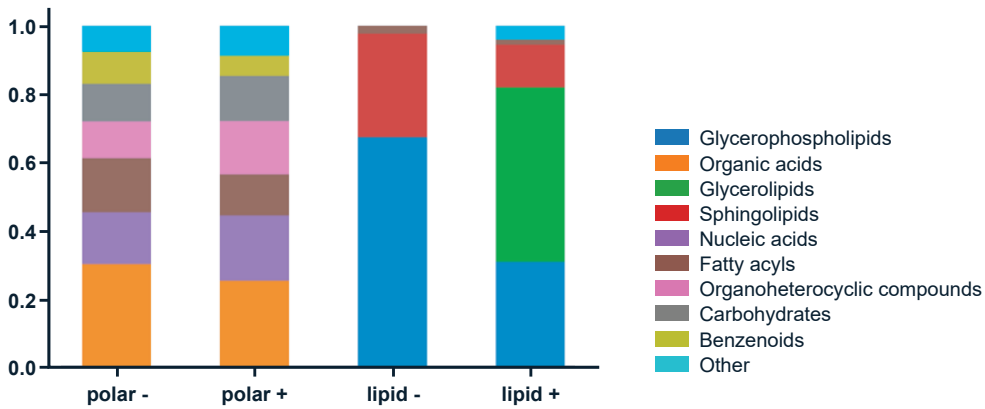


**Figure 2: Chemical composition of identified metabolites.** The chemical class composition of the identified metabolites is shown for each assay type.

## Technical variation

A summary of the technical variation across the assay's employed is shown below. Across all four assays, CV values were generally less than 10% (Figure 3). From a principal components analysis, there was no clustering of the samples based on analysis order, verifying that batch and run-order effects were successfully removed from the data (Figure 4).

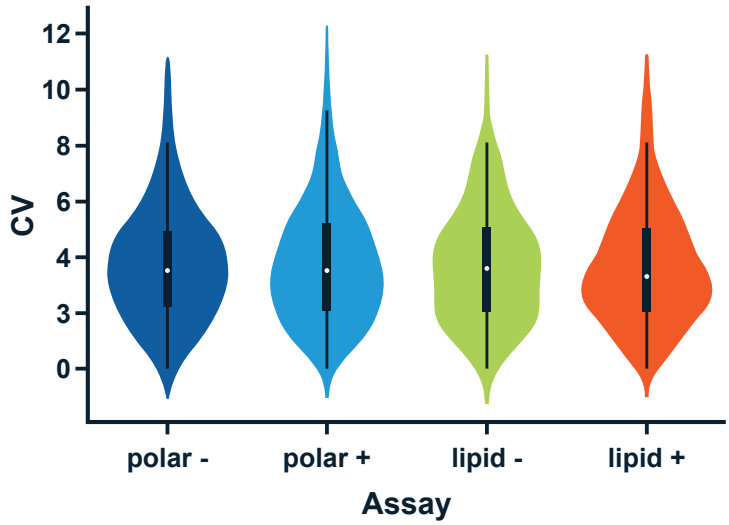


**Figure 3: Coefficients of variation for profiled metabolites.** The technical variation present in each assay is shown in the violin plot. The coefficient of variation (CV) is plotted from analysis of quality control samples.

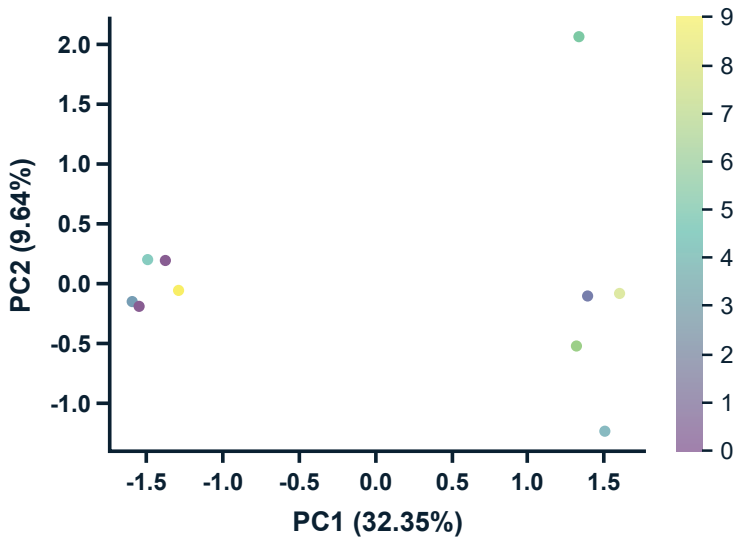


**Figure 4: Samples do not cluster base on analysis order.** The metabolic profiles (all assay results combined) for each sample are visualized in scatter plot above. Samples are colored according to analysis order.

## Metabolite associations

The global trends in the metabolite profiles of samples are visualized below in the principal components analysis (Figure 4) and heatmap (Figure 5).

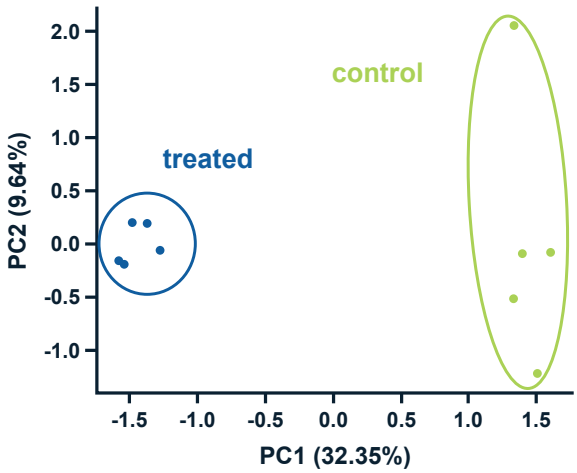


**Figure 5: Principal components analysis of metabolomics data.** The metabolic profiles (all assay results combined) for each sample are visualized in scatter plot above. Samples are colored according to experimental group.

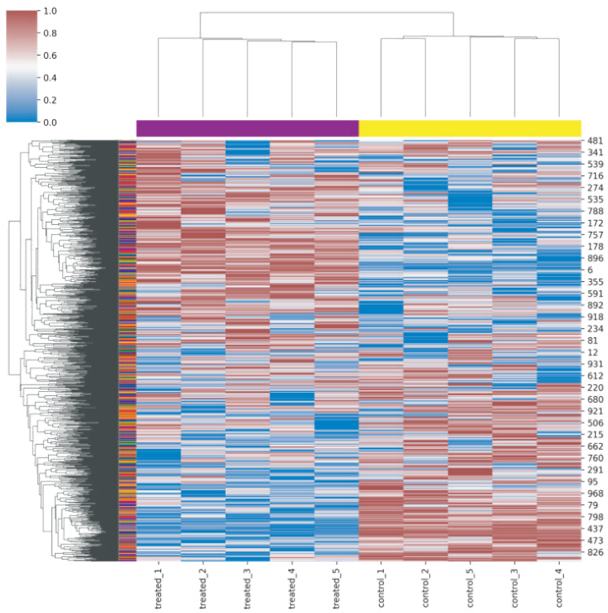


**Figure 6: Heatmap of metabolomics data.** The metabolic profiles (all assay results combined) for each sample are visualized in the heatmap above. Each column represents a sample, and each row represents a metabolite. Columns are colored according to experimental type (yellow = control, purple = treated). Rows are colored according to assay type (polar- = blue, polar+ = orange, lipid- = green, lipid+ = red).

To determine specific metabolic alternations between treated and control samples, null hypothesis testing was performed on all profiled metabolites, revealing 28 significantly altered metabolites (Figure 7). These metabolites are shown in Figure 8.

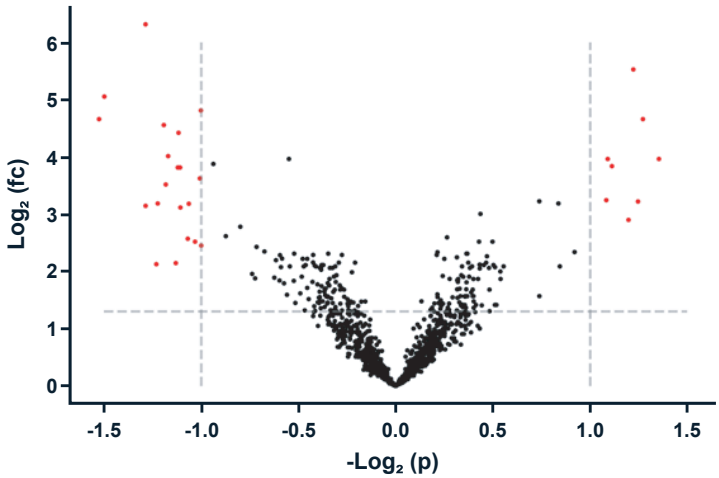


**Figure 7: Volcano plot of metabolites.** The p-values and $\log_2$ fold-changes of the metabolite levels between control and treated samples are plotted against each other in the plot above. Red dots indicate features that pass the $p < 0.05$ (false-discovery rate corrected) cutoff and have an absolute $\log_2$ fold change > 1. .

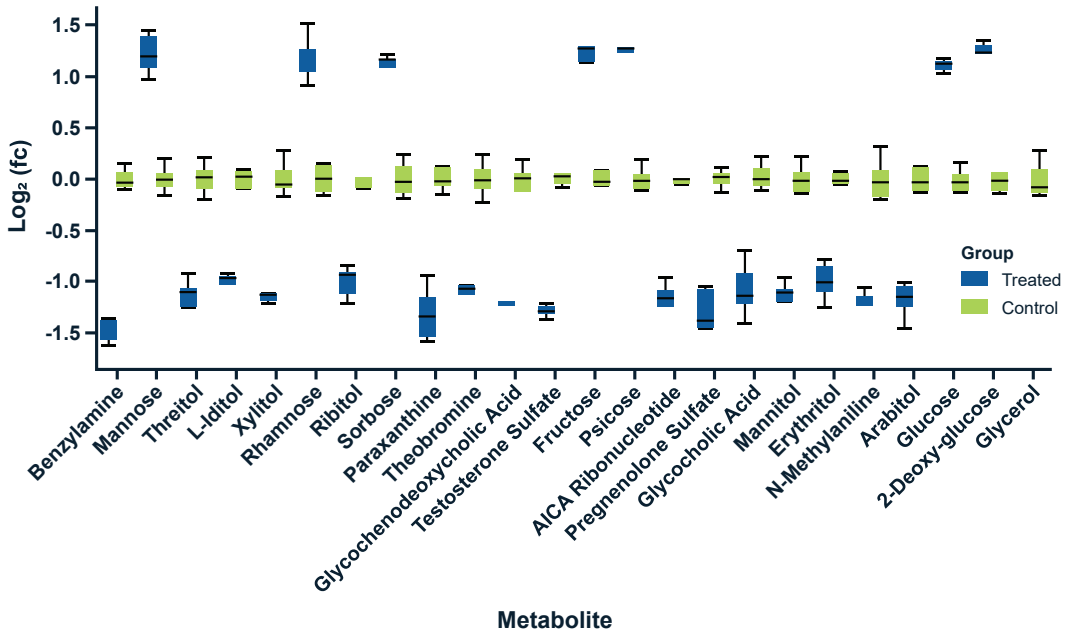


**Figure 8: Boxplot of significantly altered metabolites.** $\log_2$ fold-change of significantly altered metabolites ($p < 0.05$ (FDR corrected), $|FC| > 2$) are plotted for each experimental group.

# Interpretation

To gain biological insight from the dysregulated metabolites, an over-representation analysis was performed which identified key metabolic pathways altered between control and treated samples. These pathways include sugar (galactose, sucrose, fructose) metabolism pathways, as well as caffeine metabolism as the top results (Figure 9).

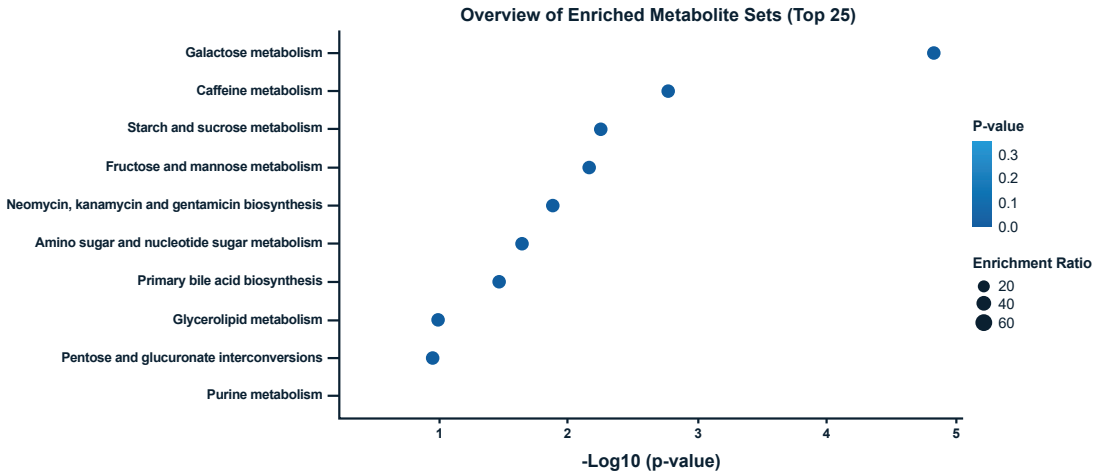


**Figure 9: Pathway analysis of significant metabolites.** The pathways found to be enriched from metabolite analysis across experimental groups is shown for each comparison.

# Appendix

## Supplementary Files

1. raw_data.zip: raw data in an open (.mzML) format.
2. Processed_data.xlxs: excel file containing sample metadata, technical variation metrics for each metabolite, all detected unique metabolite features and their raw intensities, the metabolite identifications and confidence for each feature, and the normalized and corrected metabolite abundance used for the statistical analyses.
3. Plots.zip: high-resolution images for all figures in this report as well as boxplots showing the intensity of each metabolite across all experimental conditions.

## Glossary

1. LC/MS: Liquid chromatography couple to mass spectrometry. The analytical technique used for the metabolite assays.
2. Retention time: The elution time of metabolites in the LC portion of the analysis. Retention time is determined by the chemical properties of the metabolite (e.g., hydrophobicity)
3. m/z: The mass (m) to charge (z) ratio of the measured ions in the MS portion of the analysis. In metabolomics, the charge of the metabolites is typically one or two. Thus, this can be thought of as just the molecular mass of the ions.
4. Features/metabolites signals: Signals in the raw data that represent unique compounds or metabolites measured in the LC/MS experiment.
5. Intensity: The relative abundance of the compound/metabolite in the sample.
6. Quality control sample: A sample repeatedly analyzed to measure system stability and determine signal drift.
7. Batch/run-order effect: Changes in the intensity of a metabolite signal due to the instrument drift over time.


**Contact us to initiate a project**

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